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Recombinant allergens for pollen immunotherapy

Specific immunotherapy (IT) represents the only potentially curative therapeutic intervention of allergic diseases capable of suppressing allergy-associated symptoms not only during treatment, but also after its cessation. Presently, IT is performed with allergen extracts, which represent a heterogeneous mixture of allergenic, as well as nonallergenic, compounds of a given allergen source. To overcome many of the problems associated with extract-based IT, strategies based on the use of recombinant allergens or derivatives thereof have been developed. This review focuses on recombinant technologies to produce allergy therapeutics, especially for allergies caused by tree, grass and weed pollen, as they are among the most prevalent allergic disorders affecting the population of industrialized societies. The reduction of IgE-binding of recombinant allergen derivatives appears to be mandatory to increase the safety profile of vaccine candidates. Moreover, increased immunogenicity is expected to reduce the dosage regimes of the presently cumbersome treatment. In this regard, it has been convincingly demonstrated in animal models that hypoallergenic molecules can be engineered to harbor inherent antiallergenic immunologic properties. Thus, strategies to modulate the allergenic and immunogenic properties of recombinant allergens will be discussed in detail. In recent years, several successful clinical studies using recombinant wild-type or hypoallergens as active ingredients have been published and, currently, novel treatment forms with higher safety and efficacy profiles are under investigation in clinical trials. These recent developments are summarized and discussed.

KEYWORDS: grass pollen allergy ■ immunotherapy ■ modified allergens ■ recombinant allergens ■ tree pollen allergy ■ weed pollen allergy

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At present, allergen-specific immunotherapy (IT) represents the only disease-modifying treatment for allergic diseases. However, the treatment strategy is highly dependent on the quality of the allergen product applied. Allergen extracts have been developed as active ingredients for this therapeutic intervention, yet due to their biologic origin, the molecular composition of such products can vary considerably. Given the fact that the most important allergen sources harbor more than a single allergen, it is technically tremendously challenging for allergen manufacturers to define the exact potency of their extracts. Practically, either total IgE binding of the extract or IgE binding to a very limited number of major allergens can be quantified. On the other hand, recombinant technology can provide the means to overcome such problems. Starting with the cloning of the first allergens in 1988, the production of allergenic molecules in heterologous expression systems has become a routine procedure [1–3]. The main advantages of recombinant allergens are that the molecules can be fully characterized and produced with a high batch-to-batch consistency, the reagents can be easily quantified allowing definition of

the accurate potency of a therapeutic product, and the proteins can be formulated according to the need of the patients. In other words, recombinant technology allows the production of low IgE-binding allergens with a high T cell-activating and -modulating capacity, thus leading to an improved safety and efficacy profile of allergy vaccines [4]. Moreover, the formulations contain only the relevant components, which is impossible to accomplish with allergen extracts, thus *de novo* sensitizations to other allergens, as observed in extract IT, can be excluded by careful patient selection [5,6]. Multiple sensitizations towards several allergens could in principle hinder the routine application of recombinants for therapy; however, as elegantly demonstrated for grass pollen IT, combinations of several recombinant allergens in a single vaccine preparation can be clinically effective [7].

This review will discuss concepts to generate IT vaccine candidates for tree, grass and weed pollen allergies. Candidate molecules demonstrating improved safety and efficacy profiles have been developed for many allergen sources and are in the pipeline for clinical testing (summarized in TABLE 1). For birch and grass pollen, the

Table 1. Hypoallergen concepts for pollen immunotherapy.

Allergen source	Allergen	Approximate sensitization rate (%)	Hypoallergen approaches for immunotherapy	Ref.
Silver birch	Bet v 1	60–98	Natural hypoallergens, allergen fragments, allergen trimer, trimeric fragments, allergen chimers, shuffled allergens, chemically modified allergens, mutants, mosaic allergens, overlapping peptides, allergen–carrier fusions, and peptide–carrier fusions	[10]
Olive	Ole e 1	80	Mutants and peptide–carrier fusions	[38]
Mountain cedar	Jun a 1	70	Truncated allergen	[96]
Timothy grass	Phl p 1	>90	Peptides, peptide–carrier fusions, mosaic allergen, fragments, Phl p 1–Phl p 5 fusion and Phl p 1–2–5–6 fusion	[60]
	Phl p 2	45	Phl p 2–Phl p 6 fusion and Phl p 2-truncated Phl p 6 fusion	[60]
	Phl p 5	55	Peptides, mutants, proline deletions and proline deletions plus mutations	[60]
	Phl p 6	50	Fragments	[60]
	Phl p 12	20	Tail-to-head fusion	[47]
Common ragweed	Amb a 1	>95	Fragments and peptides	[97]
Mugwort	Art v 1	95	Cysteine mutants	[97]
Wall pellitory	Par j 1	95	Cysteine mutants, loop motif mutants, deletion mutants and Par j 1–Par j 2 hybrids	[97]
	Par j 2	80	Cysteine mutants and fragments	[97]
White goosefoot	Che a 1	70	Che a 3–Che a 1–Che a 2 fusion	[97]
Russian thistle	Sal k 1	65	Mutant protein	[97]

Sensitization rates are given for people allergic to the particular source.

first successful clinical trials based on recombinant allergens could deliver encouraging results for further developments. Therefore, an optimistic view towards allergy vaccines based on recombinant technology is well supported and the first clinical products could be marketable within the next few years.

Tree pollen allergies

■ Recombinant allergens of the Bet v 1-family

Wind-pollinated trees represent a serious health problem for allergic individuals in many regions all over the world. The orders of Fagales including the families Betulaceae, Corylaceae and Fagaceae, the order of Lamiales including the family Oleaceae, and the order of Coniferales with the family Cupressaceae comprise the clinically most relevant allergy-eliciting tree species [101]. Trees belonging to the botanical order of Fagales are widely distributed within the temperate climate zone of the Northern

hemisphere. Sensitization rates vary in a range from 5 to 54%, the latter was reported for Zurich (Switzerland) [8]. Fagales allergies are mainly elicited by one family of allergens classified as pathogenesis-related protein family-10. The first described allergen of this family was Bet v 1 from birch and homologous allergenic proteins were found in pollen of related tree species. All members of the Bet v 1 family display a distinct protein structure, which results from highly homologous amino acid sequences [9]. Sensitization rates to Bet v 1 range from 62 to 100% among birch pollen allergic patients [10]. Bet v 1 homologs from hazel (Cor a 1), alder (Aln g 1), hornbeam (Car b 1), European hop hornbeam (Ost c 1), oak (Que a 1), chestnut (Cas s 1) and European beech (Fag s 1) have been produced as recombinant proteins in *Escherichia coli* and characterized extensively [9,11–13]. Moreover, a multitude of hypoallergenic Bet v 1 derivatives has been designed and recombinantly produced in *E. coli*, and several of these vaccine

candidates were evaluated in mouse models or even in clinical studies. One of the first approaches in this direction was the dissection of Bet v 1 into two nonanaphylactic fragments ranging from amino acids 1 and 74, and from 75 to 160. By disrupting the 3D structure of Bet v 1, human serum IgE binding was drastically reduced, nevertheless the ability of the fragments to activate allergen-specific T cells could be retained [14,15]. Despite the structure of Bet v 1 having been destroyed, the fragments were able to induce Bet v 1-specific blocking antibodies in immunized animals. Of note, the C-terminal fragment could skew the immune response towards Th1, as reflected by the production of the key cytokine IFN- γ , whereas the immune response elicited by fragment 1–74 was dominated by IL-4, IL-5 and IL-13 production [16]. More or less in parallel, a Bet v 1 trimer was engineered by the genetic fusion of three full-length copies of the major birch pollen allergen [17]. The fusion of several Bet v 1 molecules led to aggregation of the trimer in solution, thus it was suggested that this would lead to an altered presentation of IgE epitopes, which would explain the hypoallergenic nature of the molecule [18]. Both the Bet v 1 trimer and a mix of the two Bet v 1 fragments were successfully tested in a clinical study (which is reviewed in greater detail in the IT trials chapter of this article). Using the technology platform generated with the Bet v 1 fragments and trimer, derivatives of Bet v 1 were designed consisting of three repeats of the amino acids 1–74 or 75–160, respectively. The trimeric fragment molecules demonstrated reduced allergenic activity but induced T-cell proliferation comparable to wild-type Bet v 1 [19]. Moreover, chimeric proteins were generated by combining amino acid fragments 75–160 plus 1–74 (rBet v 1-rs1), amino acids 110–160 plus 1–109 (rBet v 1-rs2) or amino acids 110–160 plus 60–109 plus 1–59 (rBet v 1-mosaic). Patients' IgE did not bind to any of the Bet v 1 derivatives and stimulation of basophils isolated from birch pollen allergic patients revealed a strong reduction in the expression of CD203, with rBet v 1-rs2 demonstrating the lowest levels of activation. Immunization of rabbits with any of the three recombinant antigens demonstrated the induction of IgG-blocking antibodies [20]. Since birch pollen allergic patients are often multisensitized to several allergy-eliciting members of the Fagales family, gene shuffling was used to rapidly evolve the Bet v 1 allergen family aiming at the generation of a low IgE-binding vaccine candidate, which would harbor T-cell epitopes of

several Bet v 1-like pollen allergens. After several rounds of screening, two chimeric molecules, TPC7 and TPC9, consisting of sequences from the major allergens from birch, alder and hazel, were selected for full characterization. Both chimeras had lost their allergenicity, however, they still possessed a high T-cell stimulating capacity. Genetic immunization of mice with the chimeric proteins induced high levels of protective IgG antibodies and a skewing of the immune response towards Th1, as observed in ELISpot assay [21]. In 2008, Kahlert *et al.* developed a Bet v 1 fold variant by chemical denaturation of the Bet v 1 structure [22]. The structural rearrangements led to an abolishment of the IgE-binding activity of the Bet v 1 fold variant, whereas T-cell reactivity, as well as immunogenicity, was preserved [22]. Thereafter, the molecule was tested in clinical studies (Phase II and pivotal Phase III) and a follow-up multicenter, double-blind, placebo-controlled Phase II study is ongoing [23,102]. By using *in silico* structure analysis of Bet v 1 followed by mutagenesis of susceptible amino acids, Thalhamer *et al.* generated hypoallergenic Bet v 1 fold variants, which were shown to be immunogenic in mice [24]. In a similar approach, a genetically engineered fold variant of Bet v 1 was generated showing that the knowledge-based modulation of the Bet v 1-like fold resulted in a molecule with reduced IgE-binding capacity, but enhanced T cell-activating properties. The protein termed BM4 was produced in *E. coli* and, despite major structural rearrangements, still behaved as a monomer in solution. Moreover, BM4 could induce a Th1-biased immune response in a mouse model, which led to a robust induction of protective Bet v 1-specific IgG antibodies [25]. A follow-up study could even demonstrate that BM4 was taken up faster by APCs than wild-type Bet v 1, resulting in a stronger proliferation of peripheral blood mononuclear cells (PBMCs) and Bet v 1-specific T-cell clones from birch pollen allergic patients. These findings strongly suggest that the interaction of allergens with APCs might be one of the key elements to tackle for allergy vaccine design [26]. Last but not least, one has to mention that recombinant wild-type hypoallergens are also under investigation, to be used as active ingredients for birch pollen IT [27]. However, the quality of the recombinant products has striking effects on their immunologic behavior as demonstrated for the low IgE-binding Bet v 1 isoform 0102, formerly termed Bet v 1d or Bet v 1.0401. Two *E. coli* batches, one produced from the soluble fraction, the other from inclusion bodies, were

compared. Interestingly, only the protein derived from inclusion bodies demonstrated markedly reduced IgE-binding properties and, also, the fold of the allergen was modified [28].

■ Allergen-carrier fusions for birch pollen IT

Besides the modulation of Bet v 1 itself, the dissection of the molecule followed by fusion of Bet v 1-derived peptides to a carrier represents an effective strategy for vaccine design. In a first approach, Bet v 1 was dissected into six peptides with an overall length of 25–32 residues. The peptides did not bind to human serum IgE nor did they provoke allergic reactions in skin prick tests. However, when fused to keyhole limpet hemocyanin (KLH), the peptides would induce blocking antibodies in immunized animals [29]. In a slightly different approach, the fusion of two epitopes of Bet v 1 from IgE-binding regions of the allergen (termed PA and PB) to PreS from the HBV, aimed to combine an allergy vaccine with a nonallergen-related carrier providing T-cell help, again following the hapten-carrier principle. Several constructs were produced as recombinant proteins and demonstrated no IgE reactivity, as observed by ELISA using birch pollen allergic patients' sera. Immunization experiments revealed that the fusion proteins evoked a strong IgG immune response directed against the IgE epitope regions contained in the PA and PB peptides [30]. The same approach was also used to generate vaccines for grass or olive pollen allergies, whereas a carrier-grass-peptide construct is being evaluated in a clinical trial [31].

Wild-type Bet v 1 was also used as a fusion partner for the B subunit of cholera toxin and for the bacterial surface (S-layer) protein from *Geobacillus stearothermophilus* to modulate the immunologic properties of the allergen, and to obtain adjuvant effects from the fusion partners [32,33]. The Wiedermann group developed a different approach by using the major birch pollen allergen as carrier for epitopes of related pollen or food allergens. The recombinant fusion proteins were used for intranasal pretreatment of BALB/c mice and were effective in ameliorating allergy-related symptoms in the animals following allergen exposure [34,35].

■ Recombinant Ole e 1-like tree pollen allergens

Oleaceae allergies are a major health problem in Mediterranean countries, but also in areas of the temperate climate zone. The major allergens of olive (*Olea europaea*), ash (*Fraxinus excelsior*),

privet (*Ligustrum vulgare*) and lilac (*Syringa vulgaris*) all belong to the Ole e 1-like protein family, sharing approximately 90% of their amino acid sequence [36]. Furthermore, Ole e 1-like allergens carry N-linked glycans, which play a role in IgE binding and cross-reactivity [36,37]. Several hypoallergenic mutants of olive Ole e 1 generated by site-directed mutagenesis were produced in *Pichia pastoris*. Testing of recombinant proteins with Ole e 1-allergic patients' sera or sera of sensitized mice identified one mutant with a deletion of ten amino acids from the C-terminus of the allergen, which demonstrated reduced IgE-binding capacity compared with either natural or recombinant wild-type Ole e 1, however, the T-cell response remained intact [38]. Owing to the high sequence similarities between the major allergens from olive and ash, it is widely accepted that Ole e 1 could be used as a therapeutic tool for both sensitization to olive and ash pollen. In one study, Ole e 1-derived peptides were bound to KLH as a carrier. Testing of olive pollen allergic patients from Italy, Spain and Austria with this carrier-bound nonallergenic Ole e 1 peptides demonstrated a reduced IgE-binding profile. Immunization of rabbits using the peptides resulted in IgG antibodies directed against olive Ole e 1 and ash Fra e 1. Moreover, the rabbit IgGs could inhibit the binding of patients IgE directed towards these proteins [31].

■ Cupressaceae pollen allergens

Cupressaceae pollen represent a large allergen source in parts of Europe, Asia and Northern America [39]. The major allergens from Cupressaceae belong to the family of pectate lyases and polygalacturonases [39,40] and are found in pollen of several cypress species such as: Arizona cypress (*Cupressus arizonica*); Mediterranean cypress (*Cupressus sempervirens*); mountain cedar (*Juniperus ashei*); Japanese cypress (*Chamaecyparis obtusa*); and Japanese cedar (*Cryptomeria japonica*) [39]. Depending on the exposure level, the prevalence of Cupressaceae allergies ranges from 2.4 to 9.6% in the general population, whereas prevalence rates of over 30% have been reported for patients suffering from pollinosis, as determined by questionnaires and skin prick tests [8,39]. Natural Cupressaceae allergens are glycosylated and these cross-reactive carbohydrate determinants are thought to be involved in cross-reactivity between these allergens [41]. Cup a 1 was cloned, expressed in *E. coli*, purified from inclusion bodies and refolded thereafter. In circular dichroism spectroscopy the protein showed extensive β -structures, which would be in good accordance with the β -helical fold of pectate

lyases. To assay the immunologic properties of recombinant Cup a 1, ELISA and mediator-release assays were performed. The allergen reacted similarly with human serum IgE as natural Cup a 1 used as reference [42]. A very interesting approach was established to produce recombinant Jun a 1 from mountain cedar. The allergen was cloned into a tobacco mosaic virus vector and transferred to *Agrobacterium tumefaciens*. Thereafter, leaves of *Nicotiana benthamiana* were infected and Jun a 1 was purified from interstitial fluids. Plants expressing wild-type Jun a 1 suffered from necrosis following the expression of the active enzyme, whereas two truncated versions expressed in the leaves had lost this pectolytic activity. In ELISA, as well as mediator release assays, recombinant Jun a 1 showed rather similar reactivity to the natural protein; however, in inhibition assays, patients' serum IgE binding to natural Jun a 1 was only inhibited by 50% with the recombinant allergen. This suggests that not all IgE epitopes were accessible on recombinant Jun a 1 [43]. Presently, the recombinant production of allergenic pectate lyases seems problematic, which inhibits the development of therapeutic agents. Nevertheless, this family of allergens is of high clinical importance, thus it will only be a matter of time until recombinant pectate lyase allergens will be accessible for IT.

Grass pollen allergies

■ Recombinant wild-type allergens for grass-pollen IT

Specific IT of patients allergic to grass pollen using recombinant proteins is a challenging topic, because allergic reactions to grass pollen are usually triggered by more than a single major allergen. Grasses belong to the family of Poaceae, which describes the fourth largest family of flowering plants, however 95% of the clinically important allergy-eliciting grass species are clustered in the subfamilies of Pooideae, Chloridoideae and Panicoideae [44]. To date, 36 different allergen families from 16 allergy-eliciting grass species have been identified and officially acknowledged by the WHO/International Union of Immunological Societies Allergen Nomenclature Sub-Committee [101]. Pooideae usually grow in temperate climate zones and represent a large group of allergen sources with high cross-reactivity between the species [45], whereas Chloridoideae prefer tropical or subtropical habitats. Panicoideae preferentially grow in areas with tropical or subtropical climate, however some species are also endemic in temperate climate zones. Important grass pollen allergens are group 1, 2, 4, 5 and 6 allergens [46], whereas

also panallergens such as profilins show high sensitization rates beyond 20% among patients allergic to grass pollen [47]. Group 1 grass pollen allergens belong to the family of β -expansins and are found within all species of grasses showing sensitization rates between 70 and 100% among allergic patients [48], whereas group 5 allergens from the family of sweet grasses show sensitization rates between 60 and 93% [47].

In 1993, Phl p 5 was recombinantly expressed in *E. coli* [49]. In 1996, recombinant Phl p 1, Phl p 2 and Phl p 5 were characterized immunologically, showing that recombinant grass pollen allergens are well recognized by patients IgE [50]. To avoid expression problems in a bacterial host, recombinant Phl p 1 was modified at position 236 replacing cysteine by alanine using PCR mutagenesis. This resulted in a soluble, structurally intact molecule. Immunological comparison of the recombinant allergen with natural Phl p 1 with sera of grass-pollen allergic patients revealed that both molecules were immunologically equivalent [51]. Based on recombinant wild-type allergens, a clinical trial with a cocktail of five grass pollen allergens or isoforms thereof was performed in 2005. The outcomes of this study are reviewed in the clinical trials section of this review.

■ Grass pollen hypoallergens

In a similar way to tree pollen allergens, numerous approaches to generate grass pollen hypoallergens have been explored. Previously, IgE-binding areas of Phl p 1 have been identified by gene fragmentation [52]. Based on these findings, short (28–33 amino acids) synthetic peptides were designed, which represent portions of these longer B-cell epitopes. To test IgE binding, the fragments were either fused to KLH as carrier or left untreated, and thereafter tested by ELISA and skin prick test, respectively. No IgE-binding could be detected. However, when used as an immunogen in mice or rabbits, the fragments induced the production of IgG antibodies, which, in the case of rabbit antisera, could inhibit human serum IgE binding to recombinant Phl p 1 [53]. Later, a hypoallergenic variant of Phl p 1 was generated by dissecting the allergen into four fragments, which were thereafter recombined in a modified order to a mosaic protein showing the same size as wild-type Phl p 1, but as an altered structure. The Phl p 1 mosaic had drastically reduced allergenicity; however, the T-cell reactivity was preserved. Immunization of mice with the mosaic Phl p 1 resulted in the induction of specific IgG antibodies [54]. The determination of IgE binding regions of an allergen provides an elegant way to

tackle IgE epitopes allowing the design of hypoallergens. Phl p 1-specific human IgE antibodies recognizing the C-terminus of the allergen have been generated by phage display selection of antibody libraries from VH-encoding regions. Using several of these antibodies allowed the determination of an IgE-binding hot spot on Phl p 1, whereas point mutations within this region obviated IgE interactions. Employing this strategy, a Phl p 1 fragment, which could be an interesting vaccine candidate, was produced [55].

Based on T-cell epitope analyses, point and deletion mutants of Phl p 5, or combinations thereof, were generated and produced as fusion proteins in *E. coli*. Using this approach, T-cell epitopes could be left untouched while the IgE-binding activity of the mutants could be drastically diminished. Patients allergic to grass pollen showed no reaction to two deletion mutants when tested by skin prick test, however, the T-cell activating capacities investigated with specific T-cell lines and clones remained similar to wild-type controls [56]. As demonstrated for Bet v 1, the structure of Phl p 5 was analyzed using so-called Z-score calculations, which allowed the rational design of a Phl p 5 hypoallergen unable to react with patients' serum IgE [24]. Since IgE epitopes of grass pollen allergens are thought to be mainly conformational, the idea was put forward to generate hypoallergenic grass pollen allergen derivatives by deleting proline residues at structurally crucial positions. Thus, single or combination proline mutants of Phl p 5 were generated and tested in inhibition studies for their ability to bind human serum IgE. In general, single proline deletions did not affect IgE binding substantially, whereas multiple deletions resulted in proteins prone to form aggregates. However, the combination of proline deletions with a single proline to leucine substitution at position 211 resulted in a soluble protein showing native-like secondary structure elements, but with abrogated IgE-binding properties. Nevertheless, the mutant was able to activate Phl p 5 specific T-cell lines, an important characteristic for a promising vaccine candidate [57]. For Phl p 6, fragments of the allergen were generated by genetic engineering to reduce its allergenicity but retain immunogenicity. The rationale was to disrupt the main α -helical structure of the allergen, which diminished the binding capacity of patients' serum IgE. The three overlapping fragments covering the whole sequence were further tested for their ability to induce Phl p 6-specific IgG antibodies in animals. However, only one mutant (rPhl p 6 amino acids 31–110) was able to induce specific IgGs when formulated with

aluminium hydroxide for the immunization of mice. In rabbits, when formulated with complete Freund's adjuvant, all fragments were immunogenic, demonstrating the significance of the formulation for vaccine design [58].

The grass pollen profilin Phl p 12 represents another interesting candidate for grass pollen IT and, therefore, it was modified by rearrangements of non-IgE reactive fragments of the protein in a tail-to-head fusion. The recombinant molecule had lost its structure, thus IgE reactivity was reduced; however, T-cell activation and the induction of blocking antibodies in animal models remained intact [59].

A specific problem regarding grass pollen allergy is that most patients are multisensitized towards several different allergens. In fact, monosensitization towards Phl p 1 for instance has been reported for approximately 20% of children allergic to grass pollen, however, overall sensitization rates were over 90%. What is also interesting is that, although Phl p 5 showed the highest levels of specific IgE, the sensitization prevalence was still approximately 50%. Usually, patients allergic to grass pollen demonstrate very complex sensitization patterns, and predefined allergen mixes will not suffice to meet the exact needs of the patients [60]. Considering this problem of heterogeneity, the idea was put forward to combine grass pollen allergens to mosaic proteins applicable for IT. Interestingly, the combination of two grass pollen allergens (either Phl p 1 and 5, or 2 and 6) boosted the immunogenicity of those proteins compared with the single wild-type allergens [61]. In a follow-up study a hybrid combining four major grass pollen allergens (Phl p 1, 2, 5 and 6) was constructed and compared with single allergens. In summary, the hybrid had increased immunogenicity, induced blocking antibodies, but did not lose the IgE-binding properties of the fused single allergens [62]. Moreover, low IgE binding grass pollen allergen derivatives have been fused as reported for a rearranged version of Phl p 2, which was combined with a truncated low IgE-binding Phl p 6 derivative. The fusion again boosted the immunogenicity of the molecule [63]. Nevertheless, it remains to be tested whether allergen mosaics or cocktails of recombinant allergens provide a suitable alternative for extracts in grass pollen IT.

Weed pollen allergies

■ Recombinant weed pollen allergens for IT

In contrast to trees or grasses, the term, 'weed' does not describe a distinct botanical group of plants, however, it is rather used to define plants

without economic or aesthetic value. Worldwide, pollen from weed represents an important allergen source and sensitized patients, with some exceptions, typically develop allergic symptoms during late summer until autumn. Clinically, the most relevant weed species are ragweed (*Ambrosia*), mugwort (*Artemisia*), pellitory (*Parietaria*), chenopod (*Chenopodium*), Russian thistle (*Salsola*), plantain (*Plantago*) and annual mercury (*Mercurialis*). However, the major weed pollen allergens are mainly restricted to four distinct protein families, which are defensin-like proteins, Ole e 1-like proteins and pectate lyases, as well as nonspecific lipid transfer proteins (nsLTPs) [64]. Moreover, the panallergens profilin and polcalcin, which represent allergenic molecules with a ubiquitous distribution, have been recognized in weed pollen [65]. To date, 36 weed pollen allergens have officially been acknowledged by the International Allergen Nomenclature Sub-Committee International Union of Immunological Societies [101], and many of them were produced as recombinant proteins either in *E. coli* or the yeast *P. pastoris* [64]. Of note, the major mugwort pollen allergen, Art v 1, was successfully produced as a recombinant protein in *N. tabacum*, however the yields of recombinant Art v 1 obtained in either the leaves of tobacco plants or in cell cultures were insufficient to allow upscaled production [66]; therefore, unicellular organisms still represent the best choice for the production of recombinant weed pollen allergens.

■ Recombinant Asteraceae allergens & their derivatives

Besides the production of recombinant wild-type allergens, many approaches for the production of hypoallergenic weed pollen allergen derivatives applicable for future therapy have been developed. The major ragweed pollen allergen Amb a 1, which belongs to the family of pectate lyases, causes sensitization rates of up to 95% in ragweed pollen allergic individuals [64]. The protein was produced as a wild-type recombinant allergen in *E. coli* and purified from bacterial inclusion bodies. Recombinant Amb a 1 behaved quite different with regard to IgE reactivity in various experimental setups (i.e., immunoblot, ELISA and mediator-release assays), thus suggesting that a high-quality preparation of the protein would be needed for reproducible immunologic evaluation [67]. Natural Amb a 1 is proteolytically processed resulting in two fragments termed α - and β -chain. Immunoblot analyses of Amb a 1 and its cleavage products revealed that the IgE reactivity of the allergen was mainly triggered by the β -chain representing the N-terminal part of the

protein, whereas the C-terminally located α -chain harbored most of the relevant T-cell epitopes [67,68]. Therefore, hypoallergenic constructs as vaccine candidates for specific IT were developed based on the Amb a 1 α -chain. The pectate lyase Art v 6 from mugwort shares 65% sequence identity with Amb a 1 and both allergens show IgE cross-reactivity; however, Amb a 1 harbors more B-cell epitopes compared with its mugwort homolog and, also, the T-cell response to Art v 6 is more restricted [69]. Similar to Amb a 1, the recombinant production of Art v 6 in *E. coli* seems difficult, probably due to the intrinsic fold of pectate lyases, which is characterized by a parallel β -helix and stabilized by multiple disulfide bridges [70,71]. Unlike Art v 6, the major mugwort pollen allergen Art v 1 has successfully been produced in *E. coli*, even though the allergen has a complex head and tail structure, with a N-terminal defensin domain that contains four disulfide bonds and a C-terminal O-glycosylated hydroxyproline-rich domain [72,73]. Targeting the cysteine residues of Art v 1 resulted in the generation of low-IgE-binding fold variants, which still had the ability to activate Art v 1-specific T cells [74].

■ Weed pollen nsLTPs

A similar approach to Art v 1 was performed to modify the IgE-binding capacity of pellitory allergens. *Parietaria* represents the only clinically relevant allergy-eliciting weed of the Urticaceae. The plant is endemic in the Mediterranean area and can cause sensitization rates of 60–90% in some coastal regions in southern Europe [75]. The two major allergens of *Parietaria*, Par j 1 and 2, both belong to the family of nsLTPs; however, they share only 48–50% sequence identity and the cross-reactivity with other allergenic nsLTPs is limited [76,77]. Therefore, it has been suggested to use both *Parietaria* allergens as candidates for IT. The fold of nsLTPs is stabilized by disulfide bonds, thus cysteine mutants of both proteins were generated and produced as recombinant proteins in *E. coli*. For Par j 1, the destruction of three (PjC) or four (PjD) of the four disulfide bridges was most effective in reducing IgE binding, while the T-cell reactivity remained intact [78]. Moreover, PjC was evaluated in a mouse model demonstrating that immunization with PjC resulted in the induction of a robust IgG, but weak IgE immune response. Along this line, immunization with Par j 1 led to highly cross-reactive IgG antibodies against PjC, while IgE cross-reactivity was low [79]. To determine IgE-binding regions of Par j 2, the protein was first dissected into fragments and IgE binding was

analyzed by immunoblot and mediator-release assays. Except for peptide 48–76, IgE binding to peptides could be detected, which was dependent on the serum used for the assay [80]. For further evaluation, two fragments, which cover the full sequence of Par j 2, were selected and produced as recombinants in *E. coli*. In addition, disulfide bonds within the peptides were destroyed by a mutation to reduce IgE-binding properties of the molecules. Interestingly, the N-terminal fragment of Par j 2 triggered the expression of Toll-like receptors and the production of IFN- γ upon stimulation of PBMCs from atopic children. Thus, such recombinant vaccines could help to redirect the allergic immune response [81]. Since *Parietaria* allergies are triggered by both nsLTPs, a head-to-tail fusion of disulfide variants of Par j 1 and 2 was generated. The hybrid demonstrated a drastically reduced IgE interaction by retained T-cell activation, as analyzed using PBMCs from *Parietaria* allergic donors. Moreover, in a preclinical model, the hybrid protein could induce IgG1 antibodies, which demonstrated cross-reactivity with both parental allergens [82]. Building on this concept, a head-to-tail fusion of Par j 1 and 2 was designed, which had mutations in a loop region previously identified as important for IgE binding within both nsLTPs. The mutant demonstrated reduced IgE binding, but again the T-cell reactivity remained unaltered. Furthermore, the loop-mutated hybrid could induce cross-reactive IgG antibodies, as well as T-cells, in a mouse model of sensitization [83]. Moreover, fusions of full-length Par j 1 and 2, and derivatives thereof showing deletions in both fusion partners were constructed. By analyzing the secondary structure elements all fusion proteins showed increased content of random coil, which reduced the IgE-binding capacity of the molecules. T-cell reactivity was only reduced in one of the hybrids indicating that fusing Par j 1 and 2 followed by deletions within the allergens could be a suitable method for designing safe vaccine candidates [84].

Amaranthaceae pollen allergens

Allergic reactions to *Chenopodium*, a plant that is endemic in semidesert areas of Saudi Arabia, Iran and Kuwait, but also in parts of Europe and North America, are mainly elicited by the allergens Che a 1, which is an Ole e 1-like glycoprotein, the profilin Che a 2 and Che a 3, an allergen belonging to the polcalcin family [64]. To tackle the problem of a vaccine for *Chenopodium* IT, a chimeric fusion protein consisting of full-length Che a 3, 1 and 2, in that order, was produced in *E. coli*. The hybrid molecule

showed reduced allergenicity in ELISA as well as skin prick tests, yet the protein was still able to reactivate splenocytes of mice immunized with a mix of the parental *Chenopodium* allergens [85]. To assay the efficacy of the allergen chimera as a vaccine candidate *in vivo*, BALB/c mice were sensitized with a cocktail of Che a 1, 2 and 3, and challenged with the respective antigens prior to treatment with either the allergen mix, the allergen chimera, *Chenopodium* extract or phosphate buffer solution. Finally, the animals were again challenged with a mix of Che a 1, 2 and 3. Treatment with the chimera led to a significant reduction of specific IgE paralleled by an increased IgG2a production. The data were confirmed by an increase in IFN- γ and IL-10 production, plus a significantly elevated production of T-bet and Foxp3, whereas IL-13 and GATA-3 production was suppressed [86]. Besides *Chenopodium*, *Sal-sola kali* represents another important allergy-eliciting plant belonging to the Amaranthaceae family. The major allergen Sal k 1 has been classified as pectin methylesterase. To generate a candidate protein suitable for IT applications, a model of Sal k 1 was calculated based on the crystal structure of the Sal k 1 homolog from *Erwinia chrysanthemi*. Based on this model, possible B-cell epitopes of Sal k 1 were predicted, leading to the construction of a mutant with potentially reduced IgE-binding properties. After recombinant production in *E. coli*, the mutant protein was immunologically evaluated. Compared with wild-type recombinant Sal k 1 or *Salsola* extract, respectively, the mutant demonstrated reduced IgE binding leading to a reduction in allergenicity, as confirmed by a skin prick test, suggesting that the protein could be a suitable candidate for IT [87]. Nevertheless, further evaluation seems mandatory.

Clinical studies with recombinant wild-type allergens

In recent years, several clinical studies using recombinant allergens or their derivatives have been published (summarized in TABLE 2). In general, the complexity of allergen extracts can hamper the development of allergy vaccines based on recombinant proteins. Nevertheless, as discussed in the previous chapters, a multitude of novel concepts engaging this problem have been developed and some have already been tested in clinical trials. The use of recombinant wild-type allergens in IT of grass and birch pollen allergy has proven to be clinically effective. A randomized, double-blind, placebo-controlled trial with 62 subjects performed by Jutel *et al.* used a combination of five different

Table 2. Published clinical studies using recombinant pollen allergens or their derivatives.

Allergic disease	Study protocol	Intervention strategy	Number of patients	Ref.
Birch pollen allergy	DBPC	SCIT with a rec Bet v 1 trimer, or a mix of two rec Bet v 1 fragments compared with placebo	124 total, 49 [†] /37 [†] placebo, 38 [†] /19 [†] fragments, 37 [†] /28 [†] trimer	[90,91]
Birch pollen allergy	DBPC	SCIT with wild-type rec Bet v 1, nat Bet v 1 and birch pollen extract compared with placebo	134 total, 36 [†] /30 [†] placebo, 32 [†] /29 [†] nat Bet v 1, 33 [†] /28 [†] rec Bet v 1, 33 [†] /26 [†] birch pollen extract	[6]
Birch pollen allergy	DBPC	SCIT with four doses of rec Bet v 1 FV compared with placebo	37 total, 7 [†] /7 [†] placebo, 7 [†] /7 [†] 20 µg Bet v 1 FV, 8 [†] /7 [†] 80 µg Bet v 1 FV, 7 [†] /7 [†] 160 µg Bet v 1 FV, 8 [†] /8 [†] 320 µg Bet v 1 FV	[23]
Birch pollen allergy	DBPC	SLIT with rec Bet v 1 compared with placebo	112 total, 28 placebo, 84 active treatment, 16 withdrawals	[98]
Grass pollen allergy	DBPC	SCIT with mix of five recombinant grass pollen allergens (Phl p 1, 2, 5a, 5b and 6) compared with placebo	62 total, 28 [†] placebo, 29 [†] active treatment	[7]
Grass pollen allergy	DBPC	SCIT with four doses of mix of five recombinant GPAs (Phl p 1, 2, 5a, 5b and 6) compared with placebo	50 total, 10 [†] /10 [†] placebo, 10 [†] /10 [†] 20 µg GPA, 10 [†] /9 [†] 40 µg GPA, 10 [†] /9 [†] 80 µg GPA, 10 [†] /9 [†] 120 µg GPA	[88]

[†]Patients who entered the study.
[†]Patients who could be analyzed.
DBPC: Double-blind, placebo-controlled; FV: Folding variant; GPA: Grass pollen allergens; nat: Natural protein; rec: Recombinant protein; SCIT: Subcutaneous immunotherapy; SLIT: Sublingual immunotherapy.

recombinant grass pollen allergens, including Phl p 1 and Phl p 2, two isoforms of Phl p 5, and Phl p 6 in approximately equimolar concentration [7]. Active treatment, which was performed over a period of 1.5 years, led to the induction of grass pollen-specific IgG1 and IgG4 antibodies, in parallel with a suppression of specific IgE. Moreover, symptom and combined symptom medication scores were significantly improved, and the authors found significant improvement in five of seven separate categories in a rhinitis quality-of-life questionnaire. Adverse events reported in the actively treated group referred, in most cases, to local reactions at the injection site; however, severe systemic reactions, including urticaria, dyspnea and asthma exacerbation, were also reported [7]. Later, a double-blind, placebo-controlled, dose-ranging safety trial using the same cocktail of recombinant grass pollen allergens was conducted. A total

of 50 patients were divided into five groups (four active, one placebo) and actively treated groups received either 20, 40, 80 or 120 µg antigen as maximal dose following updosing, which started at 0.156 µg. In total, 13 injections were administered. Adverse systemic side effects grade I and II were reported in eight actively treated subjects, however, the reactions were evenly distributed within the groups. Conjunctival provocation tests were performed to assess treatment efficacy. Interestingly, the group treated with maximal 40 µg antigen showed the best improvement rates in this assay. Active treatment induced IgG1 and IgG4 antibodies against grass pollen, but there was quite some variation between the groups, which made it difficult to interpret the results. Moreover, active treatment had beneficial effects on late-phase reactions as determined by intracutaneous testing. A criticism of the study could be the small sample

size. Nevertheless, even high final doses of recombinant allergens did not result in severe side effects, thus encouraging larger follow-up trials [88]. For some allergen sources, such as birch pollen or cat hair, the use of a single disease-dominating allergen might be sufficient for IT. In fact, for birch pollen allergy a randomized, multicenter double-blind, placebo-controlled clinical trial, including 134 birch pollen allergic patients, was conducted to compare treatment efficacy of recombinant Bet v 1, natural Bet v 1 isolated from birch pollen and a conventional birch pollen. Patients received treatment for 12 weeks in weekly intervals, followed by monthly treatment for a period of 2 years. All actively treated patients demonstrated significant improvement in their rhinoconjunctivitis scores paralleled with reduced skin reactivity. The scores for rescue medication were also significantly reduced in actively treated groups. In addition, Bet v 1 IT resulted in the induction of specific total IgG, IgG1, IgG2a and IgG4, whereas the levels of IgE, IgA and IgG3 remained unaltered. Of note, in three cases extract IT induced *de novo* sensitizations towards the birch pollen profilin Bet v 2, and in one patient, Bet v 2-specific IgE levels were increased. Interestingly, despite the use of wild-type allergens or extracts, the number of reported side effects was similar to the placebo group [6]. Based on this study, a tablet for sublingual IT of birch pollen allergy is being developed and a first positive report on the efficacy of a dose finding Phase IIb/III study has been already published [89].

Clinical studies using recombinant hypoallergen preparations

The use of recombinant wild-type allergens has been shown to be clinically effective, nevertheless, treatment-induced side effects represent a major problem. Thus, it has been proposed to replace wild-type allergens with recombinant hypoallergens for IT. Niederberger *et al.* performed a multicenter, placebo-controlled, double-blind, parallel-group randomized trial, for a period of 12 month including 124 birch pollen allergic subjects using either a formulation of two hypoallergenic Bet v 1 fragments or a Bet v 1 trimer, showing reduced allergenicity [90]. Active treatment led to a significant induction of Bet v 1-specific IgG (mainly IgG1, IgG2 and IgG4) antibodies, which had a blocking effect on allergen-induced mediator release of basophils [90]. In a follow-up evaluation of the study, nasal and skin sensitivity before and after treatment were analyzed, showing a tolerance increase in nasal provocation tests, however, the improvement was not statistically significant and similar findings were determined

in quantitative skin prick tests. In summary, the trimer was more effective than the fragments in ameliorating Bet v 1-related allergic symptoms. When analyzing side effects, the trimer elicited more side effects than the fragments; however, treatment-associated adverse reactions elicited by the fragments were more severe. Unfortunately, the birch pollen season did not develop in all three test centers, which could have influenced the results and the number of subjects that were included in the trial was very low, thus positive therapeutic effects of the recombinant allergen derivatives might be underestimated [91]. Recently, a hypoallergenic fold/variant of Bet v 1, which was based on the recombinant wild-type protein produced in *E. coli*, has been developed through a chemical rearrangement of the allergen structure [22]. To investigate clinical safety and efficacy of the vaccine candidate, the protein was formulated with aluminum hydroxide for subcutaneous application and tested in a double-blind, placebo-controlled, dose-ranging Phase II trial including 37 birch pollen allergic patients. Four different doses (20, 80, 160 and 320 µg) were compared with placebo. All four doses of active treatment were well tolerated and led to a significant reduction of total symptom score, paralleled with a significant increase in allergen-specific IgG1. Notably, efficacy of the 80 µg dose was comparable with the 160 and 320 µg doses; however, the incidence of adverse reactions was lower [23]. Pellaton *et al.* developed an IT strategy for birch pollen allergy based on the use of synthetic peptides [92]. The concept was already successfully tested for the bee venom allergen phospholipase A2 [93] and comprised the dissection of Bet v 1 into contiguous overlapping peptides covering the whole sequence of the allergen. Mixtures of two or three peptides were tested in animal models, as well as mediator release assays with human basophils, and a final formulation termed AllerT (Anergis SA, Switzerland) was selected for a Phase I skin prick test study. The peptide mix was proven to be hypoallergenic and did not evoke allergic reactions in sensitized mice or patient emphasizing the high safety aspects of the intervention. However, T-cell reactivity needs to be demonstrated [92]. Therefore, the use of non-IgE reactive T-cell epitope-containing peptides could provide a suitable option for therapeutic applications and several approaches are under investigation [94]. Presently, vaccines based on the use of synthetic peptides for the treatment of birch, grass and ragweed pollen allergies are under investigation and the corresponding Phase I [103] or II [104,105] trials, respectively, have already been

completed. Of note, data from the study with T-cell epitopes of Amb a 1, comparing nasal and ocular symptoms after ragweed allergen challenge in a validated exposure chamber revealed a significant reduction of symptoms of actively treated ragweed allergic patients compared with placebo. Moreover, the treatment was reported to be well tolerated [95,106].

Currently, clinical trials with birch and grass pollen vaccines for sublingual or subcutaneous

IT, building on recombinant allergens or their derivatives, are ongoing and the future for pollen IT with recombinant proteins looks promising. An overview of completed or ongoing clinical trials with recombinant allergens is given in TABLE 3. Besides great advances in the development of vaccine candidates for birch and grass pollen allergies, novel recombinant molecules to tackle allergic reactions against ragweed, mugwort or pellitory are already in the pipeline.

Table 3. List of registered clinical trials with recombinant allergens or their derivatives.

Trial number	Candidate molecule(s)	Intervention strategy	Route of application	Progress
NCT00266526	Bet v 1 FV	Specific immunotherapy Phase II	SCIT	Completed
NCT00554983	Bet v 1 FV	Safety and efficacy Phase III	SCIT	Completed
NCT00841516	Bet v 1 FV	Immunological and histological evaluation of specific immunotherapy Phase II	SCIT	Completed
NCT01490411	Bet v 1 FV	Dose-range finding Phase II	SCIT	Completed
NCT00410930	Recombinant Bet v 1	Safety and efficacy Phase II	SCIT	Terminated
NCT00396149	Recombinant Bet v 1	Safety, tolerability and pharmacodynamic effects Phase I	SLIT	Completed
NCT00889460	Recombinant Bet v 1	Safety and tolerability Phase I	SLIT	Completed
NCT00309062	Hypoallergenic Bet v 1 derivative	Safety and efficacy Phase III	–	Completed
NCT01719133	Bet v 1 derived overlapping peptides	Skin test study Phase I	–	Completed
NCT01449786	Bet v 1 and Mal d 1	Efficacy Phase II	SLIT	Not yet recruiting
NCT01350635	Grass peptide/carrier	Skin test study Phase I and II	–	Unknown
NCT01445002	Grass peptide/carrier	Safety and dose finding Phase II	SCIT	Completed
NCT01538979	Grass peptide/carrier	Safety and efficacy Phase IIb	SCIT	Recruiting
NCT00309036	Recombinant grass pollen allergen cocktail	Safety and efficacy Phase III	SCIT	Completed
NCT00671268	Recombinant grass pollen allergens	Safety and efficacy Phase III	SCIT	Completed
NCT00666341	Recombinant grass pollen allergens	Dose-response study Phase II	SCIT	Completed
NCT01353755	Recombinant grass pollen allergens	Pivotal Phase III	SCIT	Ongoing
NCT01385800	ToleroMune® grass	Safety and efficacy Phase II	SCIT	Completed
NCT01385800	ToleroMune grass	Exposure unit study	SCIT	Completed
NCT01923779	ToleroMune grass	Observational study	–	Enrolling by invitation
NCT00878774	ToleroMune ragweed	Safety and efficacy Phase II	SCIT	Completed
NCT01198613	ToleroMune ragweed	Exposure chamber study	SCIT	Completed
NCT01448603	ToleroMune ragweed	Follow-up study	–	Completed
NCT01361412	ToleroMune ragweed	Identification of potential biomarkers to response to peptide immunotherapy	SCIT	Completed
NCT01398137	ToleroMune ragweed	Observational study	–	Completed

FV: Folding variant; SCIT: Subcutaneous immunotherapy; SLIT: Sublingual immunotherapy.

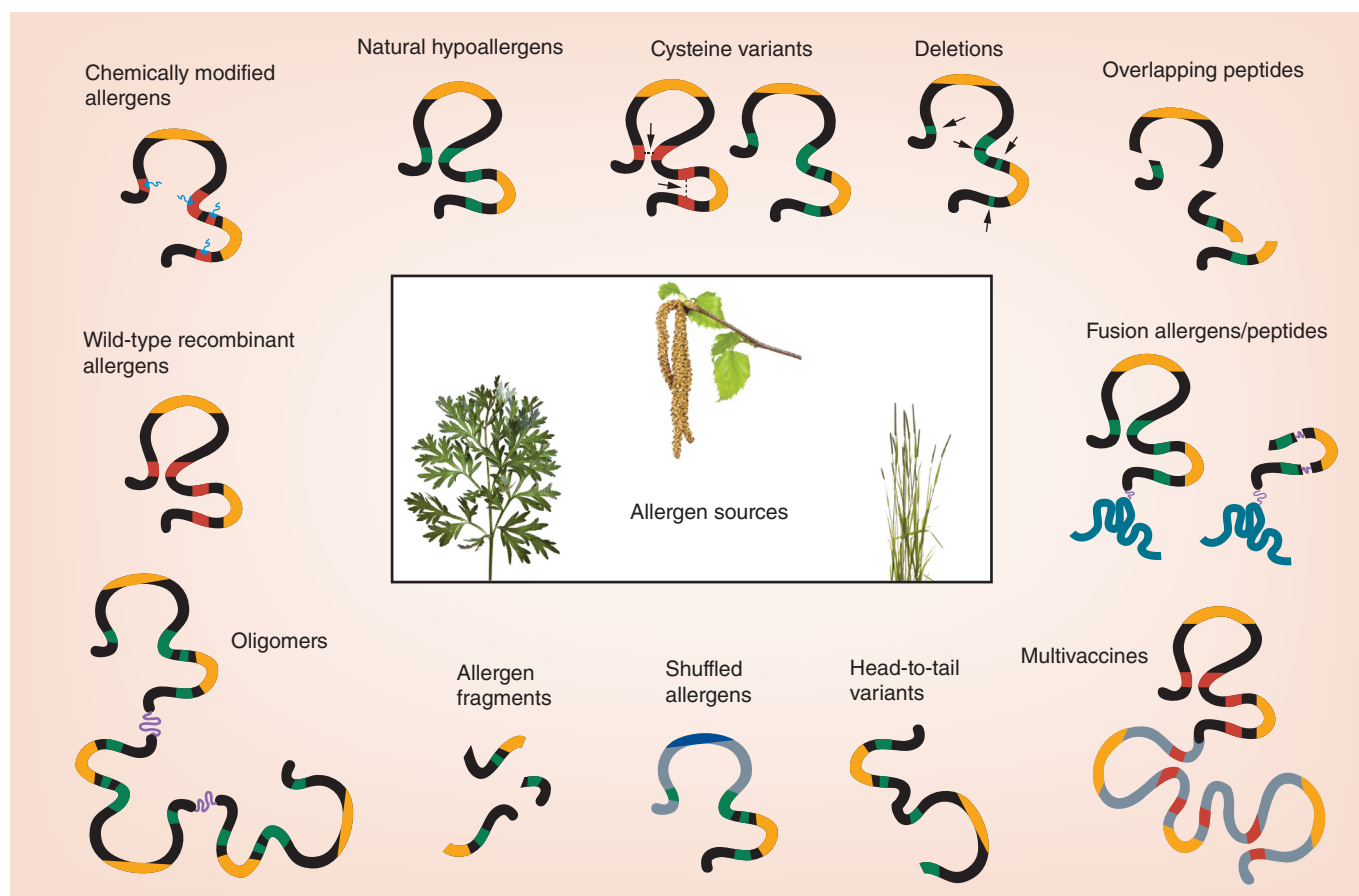


Figure 1. Concepts for the design of recombinant hypoallergenic molecules. Conformational high-affinity IgE epitopes are depicted in red, low IgE-binding epitopes are depicted in green, T-cell epitopes are shown in yellow and disulfide bridges are symbolized by dashed lines.

Please see color figure at www.futuremedicine.com/doi/full/10.2217/imt.13.114

Future perspective

The use of recombinant allergens made it possible to study the allergic immune response in detail, develop novel concepts for allergy diagnosis and, last but not least, design vaccine candidates for IT. Recombinant technology not only allows the production of wild-type allergens, but also the rational design of low IgE-binding hypoallergens. These molecules represent safe and efficacious candidates for allergy treatment. Although, most importantly, these proteins have to find their way to the clinics. In this respect, it should be emphasized that several groundbreaking clinical trials using recombinant wild-type or engineered recombinant hypoallergens highlight the potential of molecule-based strategies. As shown for the major birch pollen allergen, either recombinant wild-type Bet v 1, engineered hypoallergenic Bet v 1 fragments, a Bet v 1 trimer or a fold variant of Bet v 1 have all successfully been tested in double-blind, placebo-controlled trials. Moreover, a vaccine formulated with three contiguous overlapping Bet v 1-derived peptides

has proven its safety in clinical setup. Despite missing data on engineered hypoallergens for grass pollen, a cocktail of five major grass pollen allergens was shown to be clinically effective in IT. Moreover, for the treatment of weed pollen allergies, several interesting concepts based on recombinant allergens are in the pipeline for clinical development. Finally, one can say that there is a positive outlook for recombinant pollen allergens as vaccine candidates for IT. Nevertheless, the final proof whether a limited selection of recombinant allergens will suffice to treat complex allergies (i.e., grass pollen allergy) is still missing. Moreover, clinical studies using different formulations of IT products, as well as recombinant immune-modifying allergen-carrier fusions currently under investigation, will possibly provide interesting alternatives to conventional treatment products. Such strategies may allow a reduction in the number of doses applied, therefore reducing the duration of IT. Thus, using recombinants may help to overcome problems frequently associated with

allergen extracts, helping to improve treatment efficacy as well as safety in the long run.

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Executive summary

- Many pollen allergens of the clinically most relevant allergen sources (birch, olive, cypress and mountain cedar, grass, ragweed, mugwort, pellitory, goosefoot and Russian thistle) have been produced as recombinant proteins, most of them in the host *Escherichia coli*, but also in the yeast *Pichia pastoris*, as well as in plant-based expression systems.
- Strategies to generate recombinant hypoallergens including mutations, deletions, fragmentation, allergen reassembly, allergen fusions, allergen hybrids and chemical modifications have been developed.
- Immunotherapy using a single recombinant wild-type allergen has been shown to be clinically effective for birch pollen allergy as indicated by significant improvement of rhinoconjunctivitis scores in parallel with reduced skin reactivity.
- A cocktail of recombinant grass pollen allergens including Phl p 1 and 2, and two isoforms of Phl p 5 and Phl p 6, was tested successfully in a clinical trial with patients allergic to grass pollen, as determined by improvements in symptom and combined symptom medication scores.
- Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 showing a high safety profile have been tested in clinical trials. Bet v 1 fragments, as well as a Bet v 1 trimer, induced high levels of Bet v 1-specific IgG antibodies.
- Active treatment of birch pollen allergic individuals with a low IgE-binding Bet v 1 fold variant, led to a significant reduction of total symptom score paralleled with a significant increase in allergen-specific IgG1.

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▪ of interest

▪▪ of considerable interest

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